"BIOFABRICATION AND EXTRACTION OF NANOPARTICLES FROM SEEDS OF *Syzygium cumini*"

THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

LIFE SCIENCE

BY

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CERTIFICATE

*This is to certify that the thesis entitled "**Biofabrication and extraction of nanoparticles from seeds of Syzygium cumini**" submitted by **Ms. Stuti Pradhan** (Roll No: 412LS2054) in partial fulfilment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by her under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

Place: Rourkela

Date: 11/May/2014

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I bow my head before the Almighty for his blessings on me.

DECLARATION

I hereby declare that the thesis entitled “Biofabrication and extraction of nanoparticles from seeds of Syzygium cumini” submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the requirements for the degree of master of science in Life Science is an original piece of research work which I have carried out under the guidance of Dr. Suman Jha, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this work has been carried out or submitted to any other research institute or university or published earlier.

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LIST OF SYMBOLS AND ABBREVIATIONS USED

nm	Nanometer
mM	Milli-molar
µm	Micrometer
mV	Milli-volt
ml	Milli-litre
θ	Theta
cm^{-1}	Centimeter inverse
^oC	Degree celcius
rpm	Rotations Per Minute
min	Minute
Ag	Silver
Au	Gold
UV-Vis	Ultra Violet -Visible
DLS	Dynamic Light Scattering
XRD	X-Ray Diffraction
FE-SEM	Field Emission –Scanning Electron Microscopy
ATR-FTIR	Attenuated Total Reflection –Fourier Transform Infra-Red
SDS	Sodium Dodecyl Sulphate
SEC	Size exclusion chromatography
PE	Plant Extract

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ABSTRACT

The novel strategies are applied for synthesis of silver nanoparticles (AgNP) using biological methods, since AgNP has various applications including high anti-microbial activity. The biological method using plant extracts is relatively unexplored. Nanoparticles formed from plants are more stable and bio-compatible. For the project, *Syzygium cumini* seeds extract is used to fabricate extracellular AgNP. The ionic form of silver is reduced to its elemental form by the bioreduction reaction of silver nitrate by components present in seed extract like flavonoids, phytochemicals and alkaloids. The reduced elemental silver was further capped by the moieties present in extract into nanoparticle sizes and shapes. The sizes were further characterized for chemical, physical characteristics AgNP, using UV-Vis Spectroscope, Dynamic Light Scattering (DLS), Zeta potential, X-ray diffraction (XRD), and Scanning Electron Microscope (SEM). The morphological properties of the nanoparticles formed were spherical in shape, polydisperse, and negatively charged. Size-selective purification of the nanoparticles was done by centrifugation, filtration and chromatographic methods. In chromatographic method, elution's were collected and further characterized using UV-Vis spectroscope and Attenuated Total Reflection-Fourier Transform infrared spectroscope (ATR-FTIR).

INTRODUCTION

Nanoparticles can be defined as particles whose size ranges from 1-100 nm. These particles have a very highly surface area to volume ration, resulting in exploitation for their potential application in wide areas of both science and technology. Basically nanoparticles can be grouped under two broad categories, i.e. inorganic nanoparticles and organic nanoparticles. Generally, among the various properties, the physicochemical and optoelectronic properties [1] of metallic nanoparticles like silver, zinc, etc. are mainly dependent on their size and size-distribution. It has

been observed that the shape of these nanoparticles strongly contributes to their properties [2]. There are varieties of procedures that have been developed for the synthesis of nanoparticles, such as physical [3], chemical [4] and biological or green synthesis [5]. The physicochemical techniques for nanoparticle synthesis include methods such as photochemical reduction, laser ablation, electrochemistry, lithography or high energy irradiation, which are either too expensive or employ different substances that are hazardous to the environment, such as organic solvents, and toxic reducing agents like sodium borohydride and N,N-dimethylformamide. The chemicals used in chemical methods are often highly toxic. The surface energy of the nanoparticles is very high, thus they tend to make attractive interaction resulting into aggregate. To inhibit the aggregation, nanoparticle are need to be capped, thus additional chemical is needed to avoid coalescence and to stabilize the particles. The need of the hour is to develop some reliable, eco-friendly and non-toxic methods for the synthesis of nanomaterials. Another aspect of nanotechnology involves the synthesis of nanomaterials of various chemical compositions, sizes and morphology which also involves some suitable control over the characteristics.

There is a day by day growing need to minimize or limit the use of substances that are hazardous to the environment. The synthesis of nanoparticles using biological entities has received immense attention, and is a burning area of research since the last decade. The green synthesis [5], which is also known as the biosynthetic method, is meant for synthesizing nanoparticles using living organisms or their cellular extracts such as bacteria, fungi and plant products etc. The biological synthetic processes are simple, viable and non-toxic (if the organism/extract used is non-toxic) in comparison to physicochemical approaches to synthesize nanoparticles which are generally very toxic.

Among all the extracts, plant extracts have proved to be good biological agent to synthesize nanoparticles, particularly metal/metal oxide nanoparticles [6]. The use of plants for synthesis of nanoparticles could be advantageous over other environmentally benign biological processes, as this eliminates the intensive process of maintaining cell cultures. The effectiveness of the biosynthetic processes for the synthesis of nanoparticles would increase, if the nanoparticles could be produced extracellularly from plants or their extracts. This synthesis can be controlled in terms of their size, dispersivity and shape. This method can also be used for scale up synthesis of nanoparticles. Noble metals, especially Au and Ag, have been tested for the biosynthetic method controlling the shape and size of nanoparticles thus synthesized.

The possibility to obtain metallic particles of nanometric dimension was explored in the case of the plants and yeast. This was found when these organisms were employed for the remediation of metal-contaminated water due to a growing necessity to develop environment friendly methods to remove the toxic metals. It has been shown that many plants can actively uptake and reduces metal ions from soils and solutions during the process of detoxification. During this process metal ions are reduced into insoluble metal elemental form in the form of nanoparticles. The first successful report of nanoparticles synthesis assisted by living plants and their extracts was in the year 2002, when gold nanoparticles ranging 2 to 20 nm, was found in alfalfa seedlings[7].

Phytoremediation or the use of plants in metal extraction has appeared as a very promising alternative in the *in situ* treatment of soil and water with heavy metal ion content [8]. Eventually during this process a new method to produce metallic nanoparticles was developed. The presence of different phytochemicals confers to the medicinal [9], astringent, antimicrobial [9] and antibacterial activities of *Syzygium cumini*. The seeds are well-known to have astringent, antimicrobial and diuretic properties. Additionally, AgNP have established antimicrobial

activities[10]. Since the surface area to volume ratio of nanoparticles is very high [11], fabricated AgNP using *Syzygium cumini* seed extract can be used to enhance the individual property of adhered phytochemicals and different secondary metabolites.

REVIEW OF LITERATURE

Nanotechnology can be used to modify and engineer the properties of nanoscale materials, and structures that have become an active area of research. There are basically three methods for synthesis of nanoparticles: physical [3], chemical [12] and biological/green synthesis [5, 13]. This involves the use of bacteria [14], fungi [15] and plants [5, 16]. The growing need for developing different eco-friendly and non-toxic methods have given the direction of focus towards green synthesis of nanoparticles. The main challenges in synthesis of nanoparticles are to obtain monodispersed particles and controlling their size and shape [17]. Using plant extracts for synthesis proves to be advantageous over others as it avoids the maintenance of cell cultures and can be scaled up for rapid synthesis. It has been reported that medicinally valuable angiosperms have immense potential for synthesizing metallic nanoparticles with respect to both quality and quantity [18]. Plants may be used as whole [7] or in extracts [19, 20] for synthesizing nanoparticles.

After synthesis, nanoparticles need to be extracted from the bulk of plant extracts having flavonoids, alkaloids, proteins and different moieties. It is important to tailor low-disperse particles, as the catalytic activity of nanoparticles is dependent of the particle size and shape. For this, different purification techniques are carried out. There are many methods for the separation of nanoparticles such as electrophoresis [21], filtration [22] and chromatographic methods [23] etc. Size exclusion chromatography is a very efficient way to separate discrete sizes of nanoparticles [24].

Syzigium cumini is a therapeutic plant belonging to the family Myrtaceae of angiosperms and has many antibacterial [9], antioxidant [25] and anti-inflammatory [26] properties. The seeds are rich in many resin, albumin, alkaloids like jambosine, and many biologically active phytochemicals, like anthocyanins, antimelin and glucoside [27]. Nanoparticles synthesized from noble metals like Ag and Au have potential applications in therapeutics, bioengineering and different drug delivery systems [28]. They can be used for targeted drug delivery, detection and targeting of cancerous tissues. They do not affect the membranes of cell, while passing through them. Silver nanoparticles have antibacterial [20, 29] and antifungal [30] properties. If synthesized from *Syzygium cumini*, these nanoparticles may exhibit a very high antimicrobial activity, and even can have many additional therapeutic uses like in diabetes.

MATERIALS

$AgNO_3$ (Sigma-Aldrich, USA), SDS (Merck, India), deionised water, *Syzygium cumini* seed extracts, Sephadex G100 (Sigma, USA), Cellulose nitrate syringe filter membrane with 220 nm cut-off (Merck Millipore, USA), 2.5 μm cutoff and Isopropanol (Hi-media, India), were used for the project execution. Additional reagent used for buffer preparation or sample preparation were purchased of analytical grades.

PREPARATION OF SEED EXTRACT

Seeds of *Syzygium cumini* were collected, thoroughly washed to remove dust and impurities and shade-dried for a week. They were dried at 37ºC for two days in an incubator to remove any moisture left. They were then ground to fine powder. For synthesis of silver nanoparticles from *Syzygium cumini*, Jae Yong Song et al.[31] protocol was followed with some modifications. The powder was added in deionised water to form a suspension. Suspension was thoroughly mixed using a magnetic stirrer at 25 rpm for 10 minutes, followed by incubation in shaker incubator for 30 minutes at 37ºC. Then the mixture was centrifuged and filtered using 2.5 μm membrane filter (qualitative filter paper – Hi-media) paper. This is the desired seed extract that is needed for the bioreduction of silver ions to nanoparticles. In this method, three ratios of seed extract were prepared, 6.6:1, 3.3:1, and 1.5:1, from which 1.5:1 was optimized for the process.

Fig. (1): Preparation of Seed Extract.

1mM $AgNO_3$ solution was prepared in a 500 mL conical flask (Riviera, India), and the prepared seed extract was added according to the three different ratios. After addition of seed extract to the silver nitrate solution, instant color change in the reaction mixture was observed from a nearly colorless to yellowish brown. The color change occurs due to the reduction of silver ions to elemental form. Complete reduction usually takes 24 hours to occur for the added $AgNO_3$, but the first four hours of addition of seed extract to the silver nitrate solution are crucial as rapid reduction takes place in this time.

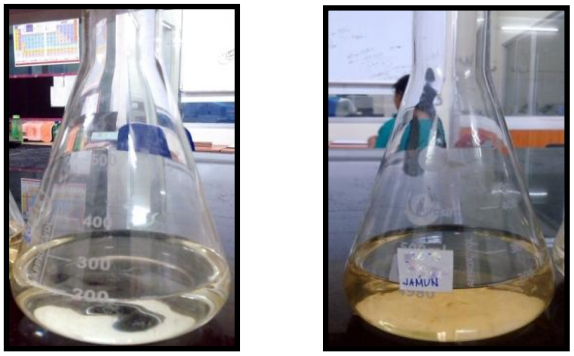


Fig.(2): (Left) Silver nitrate solution, (Right) Color changes after addition of seed extract.

CHARACTERISATION OF THE SILVER NANOPARTICLES

- **UV-VIS ABSORBANCE ANALYSIS:**

The optical property of silver nanoparticles can be studied by the absorbance they exhibit. The bioreduction study of silver nitrate solution by plant extract was monitored for 16 hours by the UV-Vis absorbance. UV-VIS absorbance analysis was carried out on a CARY-1OO UV-Vis Spectrophotometer between 300 to 500 nm at a scanning rate of 60 nm/min.

- **ATR-FTIR ANALYSIS:**

The solution was centrifuged at 12000 rpm for 20 minutes. FTIR analysis of the reaction mixture was carried out on diamond crystal, ATR-FTIR (Bruker-Germany). Scanning rate used for the analysis was 128 nm/min, for the range 500 – 4000 $c.m.^{-1}$, with resolution of 2 $c.m.^{-1}$.

- **DLS & ZETA POTENTIAL:**

The average size of the nanoparticles was determined by DLS, and their surface charge by zeta potential analysis on ZETA sizer (Nanoseries, Malvern instrument Nano Zs). The samples were diluted in deionised water followed by sonication for 15 minutes before the analysis to degas the reaction mixture.

- **FE-SEM ANALYSIS:**

To study the morphology of the nanoparticles synthesized, analysis was carried out on a FE-SEM (Nova NanoSEM 450, FEI Company, Netherland). After 24 hours of addition of seed extract to the silver nitrate solution, slides for the analysis were prepared by smearing the solution on small glass slides. As the sample is non-conductive, it was coated with a thin layer of gold just before analysis.

- **XRD ANALYSIS:**

An Analysis for miller indices was done using X-ray diffraction was carried out on RIGAKU ULTIMA IV X-RAY DIFFRACTOMETER for confirming the crystalline nature of the silver nanoparticles synthesized. Before this, the samples were lyophilized to powder form.

- **SEPARATION USING SIZE EXCLUSION CHROMATOGRAPHY:**

Sephadex G-100 was taken as the stationary phase and SDS in deionised water as the mobile phase. The optimized ratio taken as sample was centrifuged and the supernatant filtered with 220 nm filter paper. 10 mM SDS in deionised water was added to the sample and loaded to the column that was thoroughly washed in deionised water earlier. The flow rate was maintained at 1 ml/min and different elutions were collected with respect to the retention times.

RESULTS AND DISCUSSION

- **UV-Vis ANALYSIS:**

The intensity of color change occurring in the conical flask was due to the reduction of Silver ions by the plant extract. This change was measured in the form of absorbance by UV- visible spectrophotometer. There was change in peak, or peak shift in the pellet from higher to lower ratio, i.e. for 6.6:1 it was observed at 470 nm, where as for the ratio 3.3:1 it was around 450 nm.

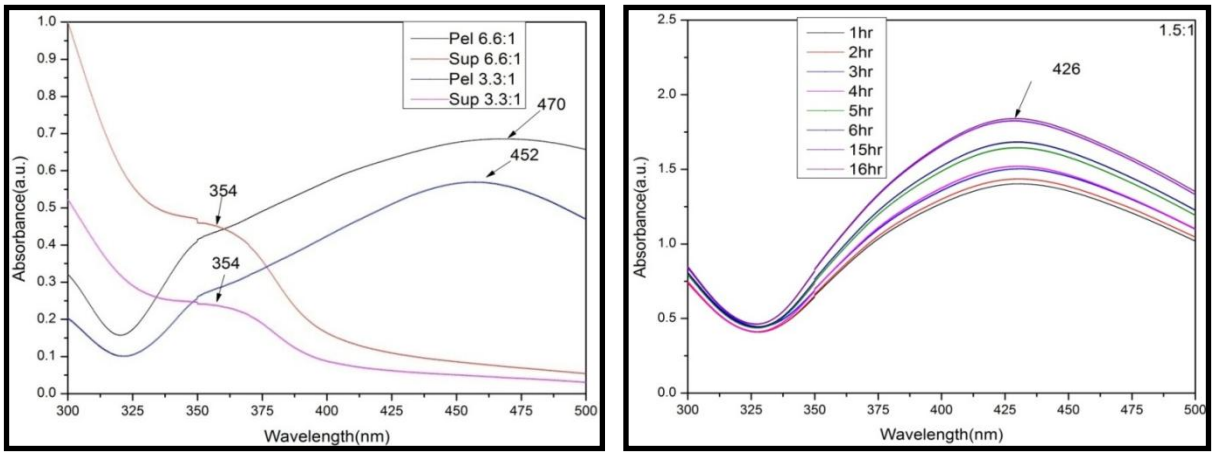


Fig.(3): Absorbance spectra of both supernatant and pellet of two ratios 6.6:1 and 3.3:1 (left), and time dependent absorbance spectra of 1.5:1 (right).

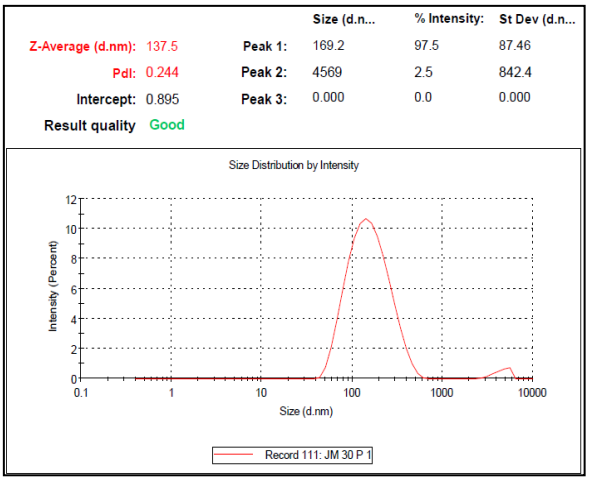
Thus, a still lower ratio of seed extract to $AgNO_3$ was prepared, i.e. 1.5:1. There was change in color, which was measured in the form of absorbance and intensity with an interval of one hour till six hours and then at sixteen hours. The maximum absorbance occurred at 430 nm and the intensity steadily increased till it got saturated after 16 hours.

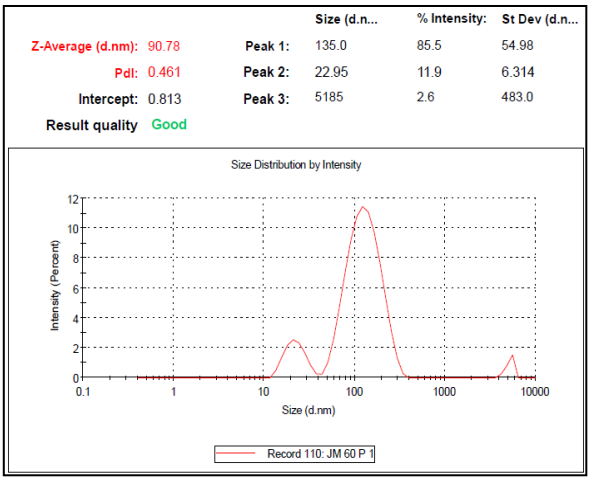
Small sized silver nanoparticles exhibit their peaks near 400 nm, where as larger sized nanoparticles tend to show increased scattering that resulting into broader peaks and shifting of the wavelength to longer wavelengths. This phenomenon is also called red-shifting. Silver

nanoparticles are known to have many optical properties [2]. These properties are dependent on the refractive index of the surrounding surface of the nanoparticles. If transferred from a denser medium to a lighter medium, the peak of absorbance of nanoparticles shifts to longer wavelengths. In another case, if nanoparticles are transferred from lighter to denser medium, the peak of the absorbance shifts to shorter wavelengths, which is also called blue-shift or banthochromic shift. Unstable particles tend to decrease the intensity of absorbance and broadening of the peak due to the formation of polydisperse or various size aggregated nanoparticles.

- **DLS ANALYSIS:**

This characterization was carried out to determine the average sizes of the nanoparticles. The average particle size of crude plant extract and nanoparticles was found to be different for different ratios (table 1, below). For the ratio 3.3:1, the average size was 137.5 nm. The particles were polydisperse, but the polydispersity of the ratio 1.5:1 is observed to be the least as compared to the other two ratios. The less the polydispersity, the better it is. As the main aim is to synthesize or extract monodisperse nanoparticles, the ratio 1.5:1 was optimized for further characterizations.





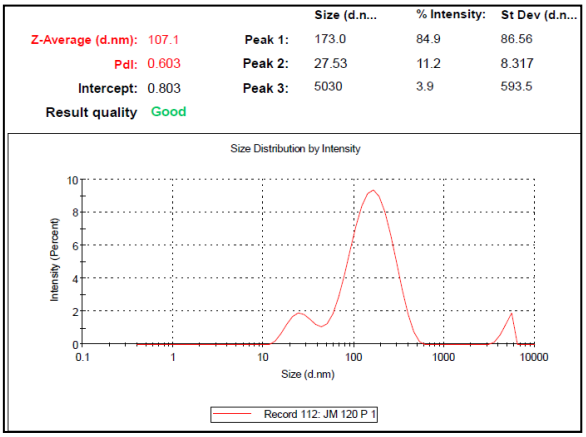


Fig. (4): DLS analysis of PE: AgNO3 (1.5:1) (top left), PE: AgNO3 (3.3:1) (top right), PE: $AgNO_3$ (6.6:1) (bottom left).

Table.1: DLS Analysis of PE: AgNO3 and their Average Size.

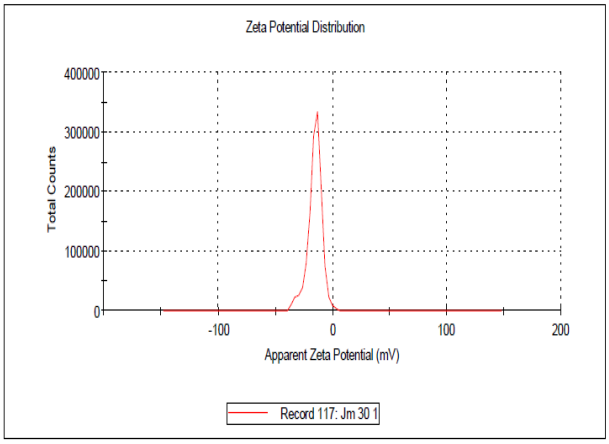
DLS analysis of PE:$AgNO_3$	**AVERAGE SIZE(nm)**
1.5:1	137.5
3.3:1	90.78
6.6:1	107.1

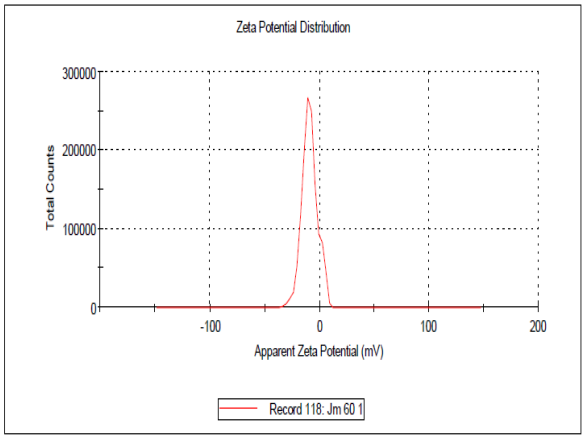
➢ ZETA POTENTIAL ANALYSIS:

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential analysis was done to determine the charge on the nanoparticle surface. This charge can be used to analyze the stability of these particles. The zeta potentials observed in the three ratios were -15.3, -8.77 and -7.92 mV for the ratios 1.5:1, 3.3:1 and 6.6:1, respectively. Thus, the stability of nanoparticle formed in ratio of seed extract to $AgNO_3$ 1.5:1 has the maximum stability as compared to the other two ratios as it has the maximum zeta potential value (from reference table 2).

Table (2). Ratio of seed extracts and their zeta potential value.

RATIO OF SEED EXTRACT:$AgNO_3$	**ZETA POTENTIAL VALUE**
1.5:1	-15.3
3.3:1	-8.77
6.6:1	-7.92





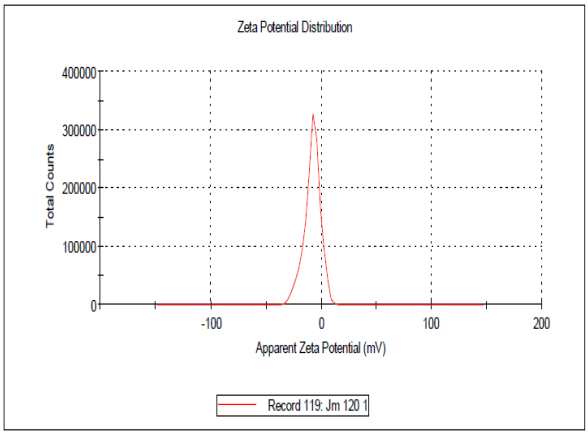


Fig. (5): (top left) Zeta potential Analysis of PE: AgNO3 (1.5:1), (top right) Zeta potential Analysis of PE: $AgNO_3$ (3.3:1), (bottom right) Zeta potential Analysis of PE: $AgNO_3$ (6.6:1).

Table.(3) Zeta value Range and their stability.

ZETA VALUE	STABILITY
0 to ±5	Rapid coagulation
±10 to ±30	Incipient instability
±30 to ±40	Moderate stability
±40 to ±60	Good stability
More than ±61	Excellent stability

➢ FE-SEM IMAGE ANALYSIS:

FE-SEM image analysis was carried out to study the morphology of the nanoparticles synthesized. The slides were prepared after about 24 hours of complete synthesis of nanoparticles. A thin film of the solution was smeared on a glass slide and left to dry. Just before the image collection, samples were coated with a thin layer of gold to impart it a conductive nature.

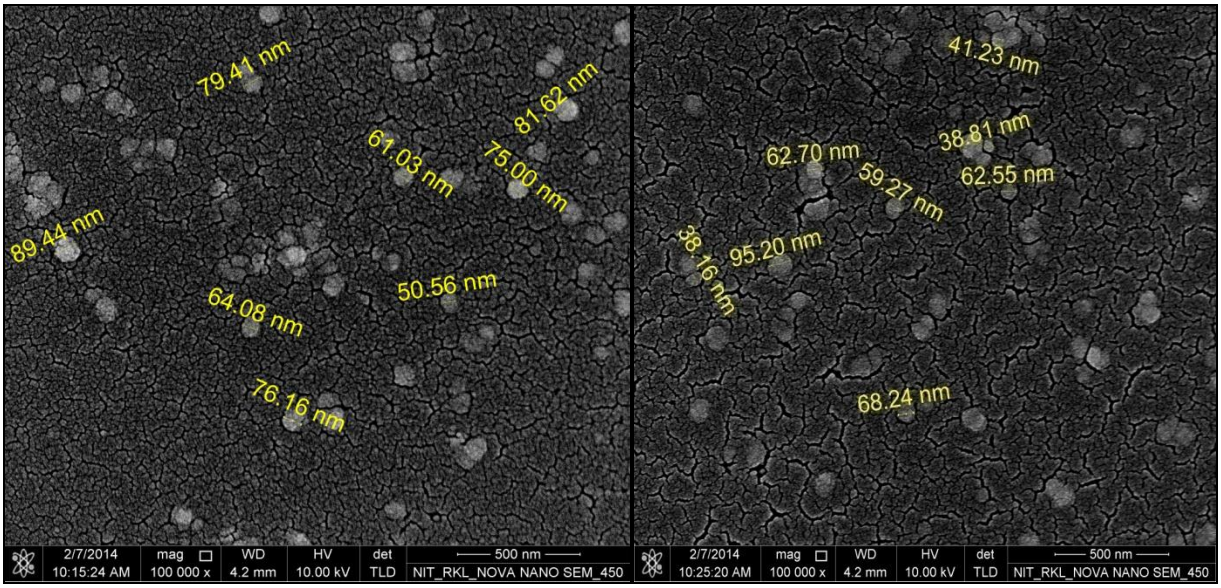


Fig. (6): FE-SEM image of the silver nanoparticles showing different sizes of spherical shape.

From the FE-SEM image it was observed that nanoparticles of different sizes have been synthesized. The shape of the dispersed nanoparticles formed is spherical.

➢ X-RAY DIFFRACTION (XRD) ANALYSIS

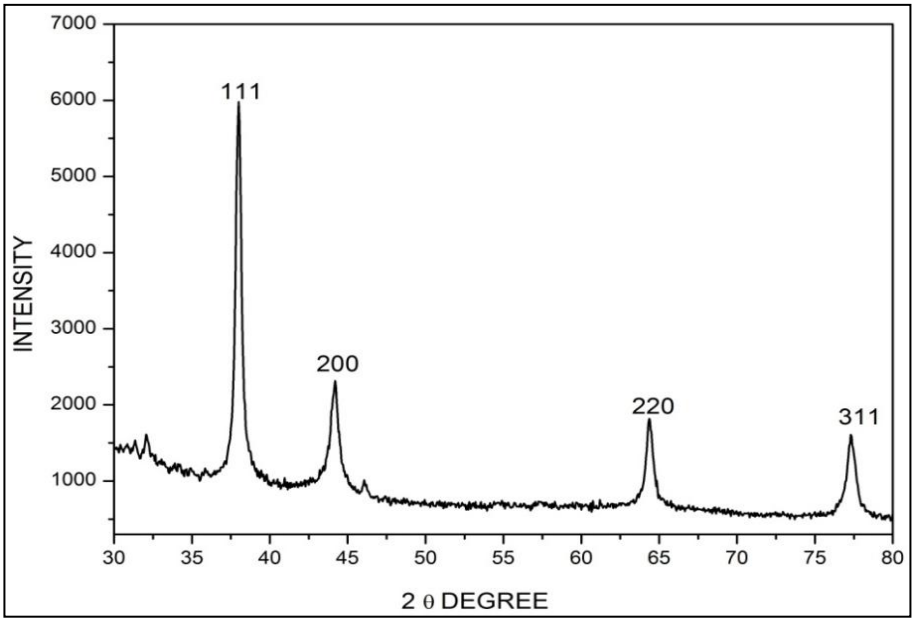


Fig. (7). XRD graph of AgNP showing sharp and narrow peaks showing crystalline nature of nanoparticles.

In fig. (7) above, the XRD patterns of silver nanoparticles synthesized from seed extract of *S. cumini* are clearly seen. A number of Bragg reflections with 2 θ values of 37.96º, 44.131º, 64.34º and 77.40º correspond to the (111), (200), (220) and (311) sets of lattice planes are observed [32]. The indices may be indexed as the band for Face Centered Cubic (FCC) structures of silver. The peaks observed were very narrow and sharp. The XRD pattern thus clearly illustrates that the silver nanoparticles synthesized by the present green method are crystalline in nature.

➢ SEPARATION OF NANOPARTICLES USING MEMBRANE FILTRATION AND SIZE-EXCLUSION CHROMATOGRAPHY:

As the UV-Vis analysis of pellet from the ratio 1.5:1 showed absorbance at 430 nm, it can be ascertained that the silver nanoparticles formed are present. Thus, membrane filtration followed by size exclusion chromatography of the pellet of this ratio was done to separate nanoparticles and purify them. For size exclusion chromatography as in fig. 8, Sephadex G-100 was taken as the stationary phase in the column, and a 10 mM sodium dodecyl sulphate in mobile phase [24]. The column was thoroughly washed with deionised water, and then the sample was placed onto the top of the column bed.

The principle of size-exclusion chromatography is that particles of different sizes will elute through a stationary phase at different rates. Particles of same size should elute together. The bigger sized molecules will elute faster than the smaller ones. The flow rate was maintained at 1 ml/min, and the elutions were collected after every 10 minutes. As the sample passes through, it leaves a turbid yellowish color behind. Precautions were taken so that the gel surface should not be exposed to dry. SDS is gently pipetted onto the column until the column is colorless again. The column is again washed with deionized water till it's free of any impurities like plant extract, nanoparticles or SDS.

Fig. (8): Set-up for size exclusion chromatography.

CHARACTERIZATIONS OF THE ELUTIONS OBTAINED FROM SIZE EXCLUSION CHROMATOGRAPHY

The elutions obtained were characterized for confirming the presence of AgNP and determining their stability.

- **UV-Vis ANALYSIS:**

The spectra from 300 – 800 nm for first nine elutions were taken, and it was observed that there was an absorbance maximum at a constant wavelength of 423 nm for the first five elutions (Fig.(9)).

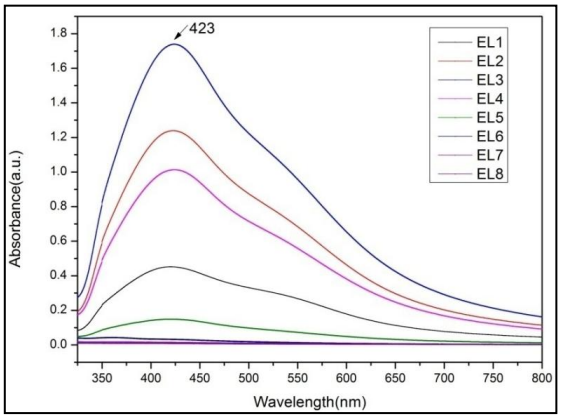


Fig. (9): UV-VIS spectra of different elutions collected from size exclusion column

The absorbance at 423 nm of the following elution decreased after the third elution. Thus, it can be concluded that the maximum population of silver nanoparticles are present in the third elution obtained (Fig. (9)). There are varied types of particles present but almost about the same size.

Different elutions contain different amount and size of silver nanoparticles, which is directly proportional to the intensity of absorbance. The absorbance of the elutions after the third one decrease, showing that the population of nanoparticles decreases and finally in the fifth elution onwards, there is negligible absorption at 423 nm. This shows that there are no nanoparticles in

them, and SDS or deionised water (mobile phase), i.e. the components with which the column is washed after size exclusion chromatography are only present.

- **ATR-FTIR ANALYSIS:**

It is observed that the silver nanoparticles solution is extremely stable for nearly 65 days with only insignificant aggregation of particles. ATR-FTIR spectroscopy measurements are carried out to identify the biomolecules that bound specifically on the silver surface. When a beam of infra-red is incident on the sample, bonds at specific regions vibrate. These vibrations can be used to identify the different biomolecules or phytochemicals that are attached to the surface of silver nanoparticles even after the size exclusion chromatography.

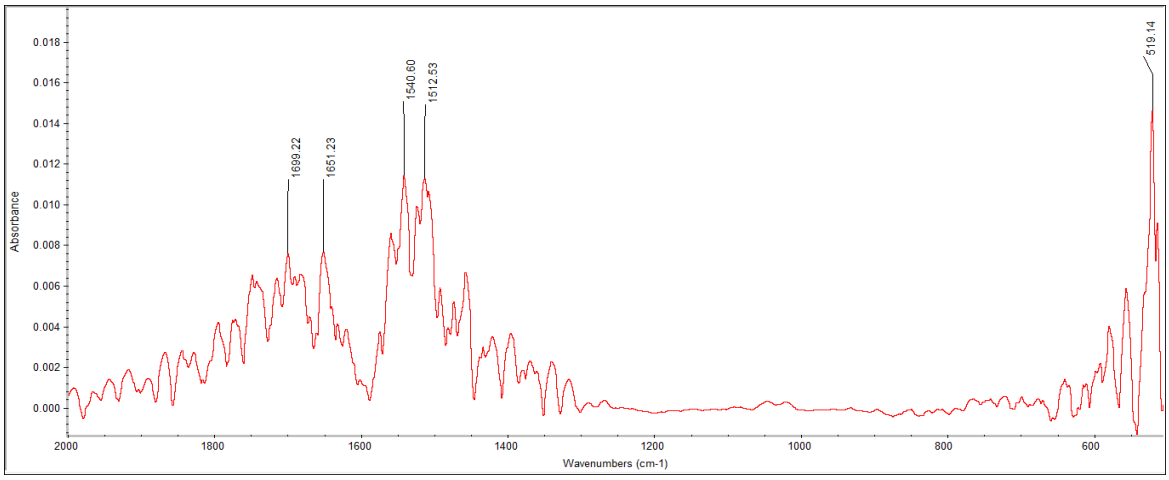


Fig. (10): ATR-FTIR analysis of elution after size exclusion chromatography.

Fig. 10 above shows the presence of bands at 519.14, 1512.23, 1540.60, 1651.23 and 1699.22 cm^{-1}. The strong absorption at 519.14 cm^{-1} is due to the nanoparticles. The bands at 1512.23 cm^{-1} are due to the C=O of different polyols present in flavonoids and other plant components like terpenoids, etc. The peak at 1540.60 cm^{-1} may attribute to the amide-II bonds. The peaks at 1651.23 and 1699.22 cm^{-1} are due to amide-I bond present in motifs and domains of biomolecules present on AgNP interface [33].

CONCLUSION

Plants have been well established for medicinal and aesthetic values. The extracts of plants have been used since time immemorial to treat various ailments. *Syzygium cumini* has many economic and medicinal values. The method of using plant extracts as reducing agents for the biofabrication of nanoparticles is very economical, rapid and reliable. Plant extracts have high reducing potential, and these act as capping agents too. The synthesis of silver nanoparticles and their conjugation with the biomolecules present in *Syzygium cumini* seed extract can prove to be of much importance and advantageous in studying the interactions with other proteins or biomolecules. The nanoparticles synthesized from the seed extract of *Syzygium cumini* were of the average size of around 100 nm. But after purification, the extra impurities were removed from them and only the useful components of plant extracts remain conjugated to the nanoparticles. These conjugated components need to be characterized for further studies on the properties of the nanoparticles to be studied in interactions with different biomolecules and their applications.

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